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Some Histopathological Findings in Dead Kihansi Spray Toads in Captivity

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Abstract

Raising of Kihansi spray toad (KST), *Nectophrynoides asperginis*, in captivity is associated with disorders that can be fatal. Since diagnosis of these disorders cannot be confirmed grossly, the current study was aimed at exploring histopathological findings in dead KSTs kept under captivity in Tanzania. Dead KSTs were immediately recovered, observed for gross changes, fixed in 10% neutral buffered formalin, processed routinely, and stained sections reviewed for histological changes. Observed infectious agents were strongyloides (25.3%), ciliates (1.7%), lungworms (0.6%), fungi (3.4%) and bacteria (5.1%) or mixed infections (9%) of these agents. Noted histopathological lesions in affected organs included infiltration of inflammatory cells, thickening of the epithelium, organ dilation, accumulation of organisms and dead tissue debris in the organs, hyperkeratosis, parakeratosis, and sloughing of the skin. Squamous metaplasia in various organs was the commonly observed non-infectious abnormality noted in 33.1% of the carcasses. It is concluded that there are several histological changes caused by infectious and non-infectious agents that are potential contributors of KST death in captivity.

Keywords: Kihansi spray toad, parasites, pathology, captivity, immunity

Introduction

In 1993, the government of Tanzania commissioned construction of lower Kihansi hydropower plant (LKHP). The construction diverted 90% of the water flow away from the Kihansi gorge leaving 1.6-1.8 m^3/s water flowto sustain the life of flora and fauna downstream the dam (Channing et al. 2009). The reduced water flow enabled close access to wetlands in the gorge that led to discovery of the Kihansi spray toad (KST), *Nectophrynoides asperginis* during implementation of environmental management plan for the LKHP project. This toad was endemic only to Kihansi gorge at the base of Kihansi river waterfalls (Menegon et al. 2004). It is a small sized animal of not more than 2.5 cm, live-bearing and insectivorous.

Not long after its discovery, the population of KST alarmingly declined and threatened the survival of the toads. In fear of extirpation, artificial sprinklers that mimicked the natural water sprays were instituted to complement the reduced water flow and support KST's survival (Channing et al. 2009). Additionally, 500 toads were translocated to six zoos in USA in 2001. Unfortunately, the number of KSTs kept going down and the population crashed in 2003. The toads were last seen in their natural habitat in 2004 (Channing et al. 2009). In 2009 they were declared extinct in the wild. Although the actual causes of extinction remain fussy, reduced water flow, collapse of sprinkler system, infections with chytrid fungus, flushing of sediments from the dam, and human activities using pesticides upstream the dam have been suggested to play roles (Channing et al. 2009). The available KSTs are descendants of 70 animals kept in captive facilities since 2001. Life in captivity might have primed adaptive attributes different from original steady states acquired in the wild. This may have impacts on the

immune responses due to limited resource diversity and genetic variability.

Food quality for animals with a wide breadth of diet composition is likely an important factor that determines infection risks (Knutie et al. 2017a). The keeping of KSTs in captivity limits the diversity of nutritional resource availability which can alter host resistance against infectious agents (Sternberg et al. 2012, Howick and Lazzaro 2014). Such resistance emanates from the ability of the immune response to reduce the damage that parasites cause. Only hosts in good conditions may be physiologically able to invest in this immunity (Lochmiller and Deerenberg 2000, Demas 2004). Since KSTs in captivity are limited in nutritional diversity and possibly higher food availability, they have fewer resources for immunity that may compromise host resistance to infections (Knutie et al. 2017b). Besides nutritional diversity, genetic diversity of adaptive immunity driven by major histocompatibility classes (MHCs) contributes to disease resistance in frogs (Bataille et al. 2015). Responses of individuals to infections vary greatly with the capacity of their immune systems to respond to the pathogens. This is a function of diversity of immune factors, including MHCs, that is genetically driven. Being descendants of 70 individuals, the captive KSTs have most probably reduced the diversity of immune factors like MHC and thereby lowered the immune responses to parasites. These facts allude to the possibility that diseases which are not fatal in natural habitats would kill captive animals.

Owing to their small sizes and restricted personnel access to the cages in captive facilities, some clinical signs and most morphological abnormalities in KSTs may go unnoticed. As a result, most KSTs are often found dead in their cages compelling for thorough histopathological examinations. The aim of the current study was therefore, to analyze pathological changes and diagnose disease conditions contributing to the KST deaths in captivity.

Materials and Methods

Study area

The study was conducted in captive facilities at the University of Dar-es-Salaam (UDSM) and Kihansi Laboratory in Tanzania.

The toads

The KSTs used in this study were descendants of toads sent to USA in 2001 from Kihansi gorge during rescue measures against extinction. They were kept in 40 x 30 x 50 cm cages at UDSM and Kihansi captive facilities. The toads were sprayed with filtered water continuously for 8 hours from 8:00 am to 4:00 pm and intermittently for 30 minutes on-off cycle from 4:00 pm to 8:00 am. Spraying was stopped for 2-3 hours during feeding that was done every fortnight. Light was provided from 8:00 am to 4:30 pm and temperature maintained at 16–19 °C. Adult KSTs were fed with drosophila or crickets, while newborns and juveniles were fed with collembola. The study was conducted from August 2010 to August 2015.

Handling of KSTs

Any dying KST from the University of Dar es Salaam and Kihansi captive facilities was fixed with 10% neutral buffered formalin and submitted to pathology laboratory at Sokoine University of Agriculture (SUA). The toads were then observed for any gross abnormalities, cut longitudinally and processed routinely as done before (Malago and Nondoli 2008, Malago and Sangu 2015). The procedure involved dehydration in graded alcohol (70%, 90%, 95% and absolute), clearing in chloroform and impregnation and embedment in molten (56 °C) paraffin wax. Processed samples were sectioned with rotary microtome to obtain 4 µm thick slices that were deparaffinised on microscopic glass slides before staining with hematoxylin and eosin (H&E). After mounting, the slides were reviewed and digital photomicrographs taken using a standalone system (SAL) of DP21 microscope digital camera (Olympus, Japan)

mounted on Olympus BX41 light microscope (Olympus, Japan).

Data analysis

Collected data were subjected to descriptive statistics and presented as percentage of cases and microphotographs of affected tissue sections from dead KSTs.

Results

A total of 178 KST carcasses were examined, of which 139 were diagnosed with different disease conditions, while 39 (21.9%) had no obvious diagnostic lesions. As shown in Figure 1, both infectious and non-infectious conditions were observed. Infectious conditions included helminthosis predominated (25.3%) by nematode worms particularly strongyloides species (strongyloidiasis) followed by bacterial infections (5.1%), fungi particularly chytridiomycosis (3.4%), ciliate parasites (1.7%) and lung worms (0.6%). The strongyloides were found in intestinal lumen from where they could spread and disseminate to various layers of the intestine causing thickening of the intestinal wall and infiltration of inflammatory cells (Figures2A, 2B and 2C). In the lungs, lungworms caused lung dilation (Figure 3A), inflammatory reactions (Figure 3B) and thickening of the lung epithelium (Figure 3C). Another parasite, ciliate protozoan, was observed to disseminate throughout the body without causing pathological changes (Figure 4).

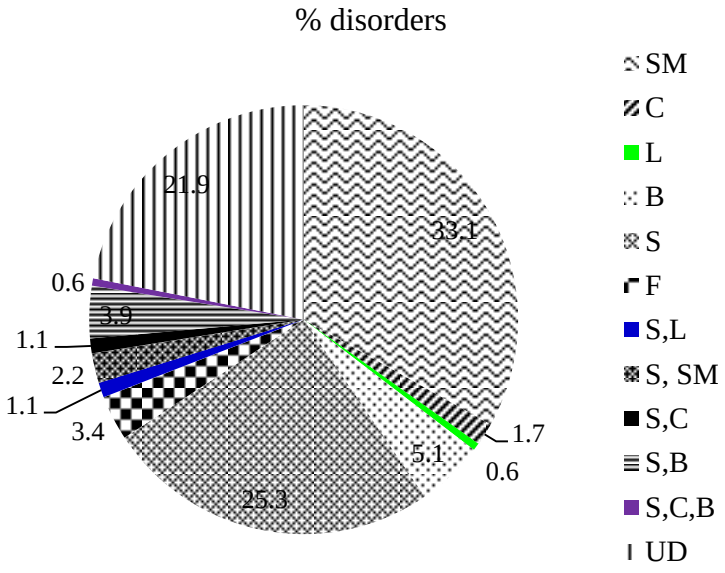
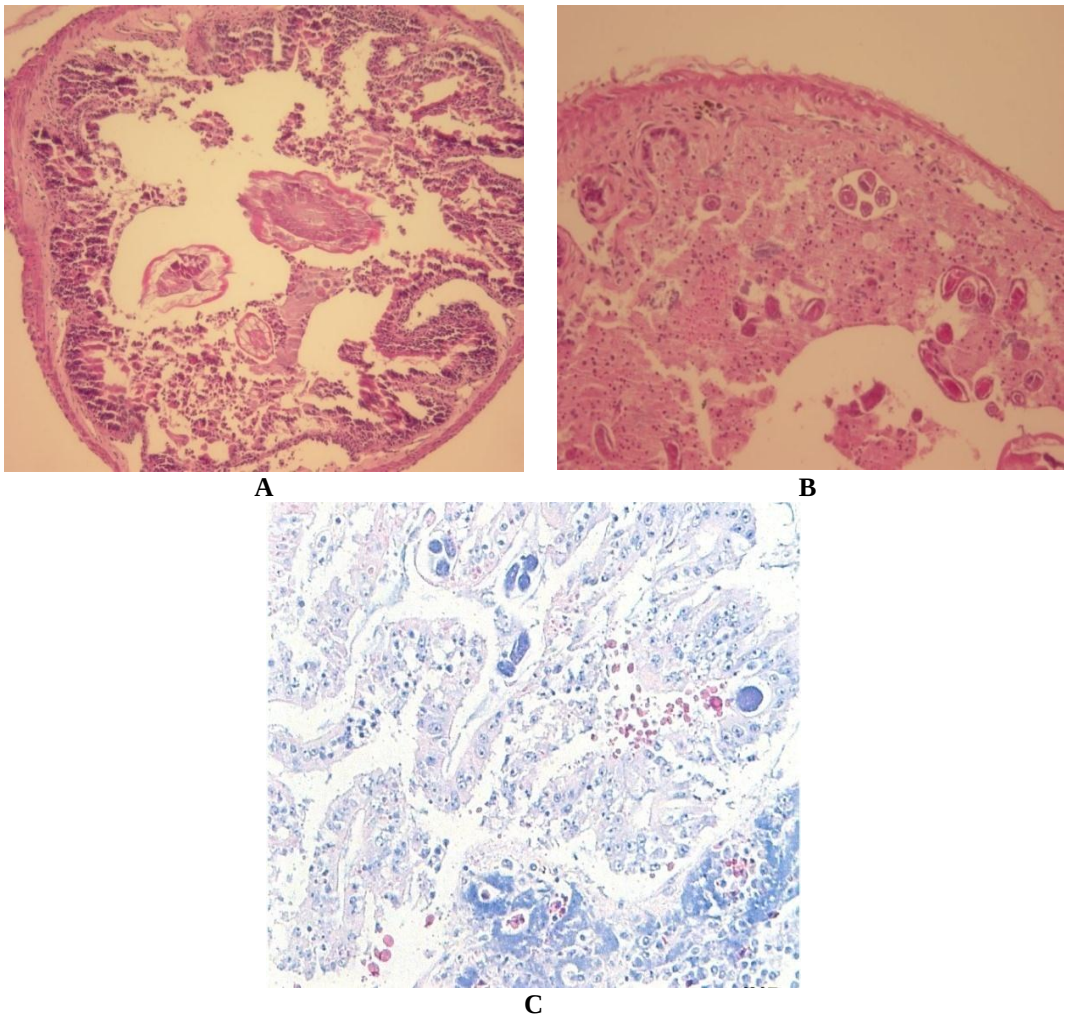


Figure 1: Disorders encountered in KSTs carcasses following histopathological examination. SM = squamous metaplasia; C = ciliate parasites; L = lung worms; B = bacterial infection; S = Strongyloides; F = fungal infection; UD = undetermined causes; N = 178.

Unidentified fungi and bacteria were observed to infect the KSTs in various tissues and organs including the skin and heart. The infections led to tissue reactions and swellings with accumulation of organisms and dead tissue debris in the organs (Figures 5 and 6).

Infection of KST with chytrid fungus was observed to affect the epidermis layer of the skin causing excessive thickening of the epidermis (hyperkeratosis) and retention of epidermal nuclei in the keratin layer (parakeratosis). Sloughing of the keratin layer

with numerous empty sporangia in different stages of parasite development was also observed (Figure 7). The stages included zoosporangia containing spores, empty sporangia that have released their spores, a divided sporangium depicting colonial sporangium or thallus, and prolific stage seen as four or more sporangia together. Most lesions occurred in the ventral skin.



A **B**

C

Figure 2: KST infected with nematodes. The nematode parasites (Strongyloides) in the intestinal lumen (A) can disseminate to intestinal wall and induce infiltration of inflammatory cells in the intestinal mucosa leading to enteritis (B and C).

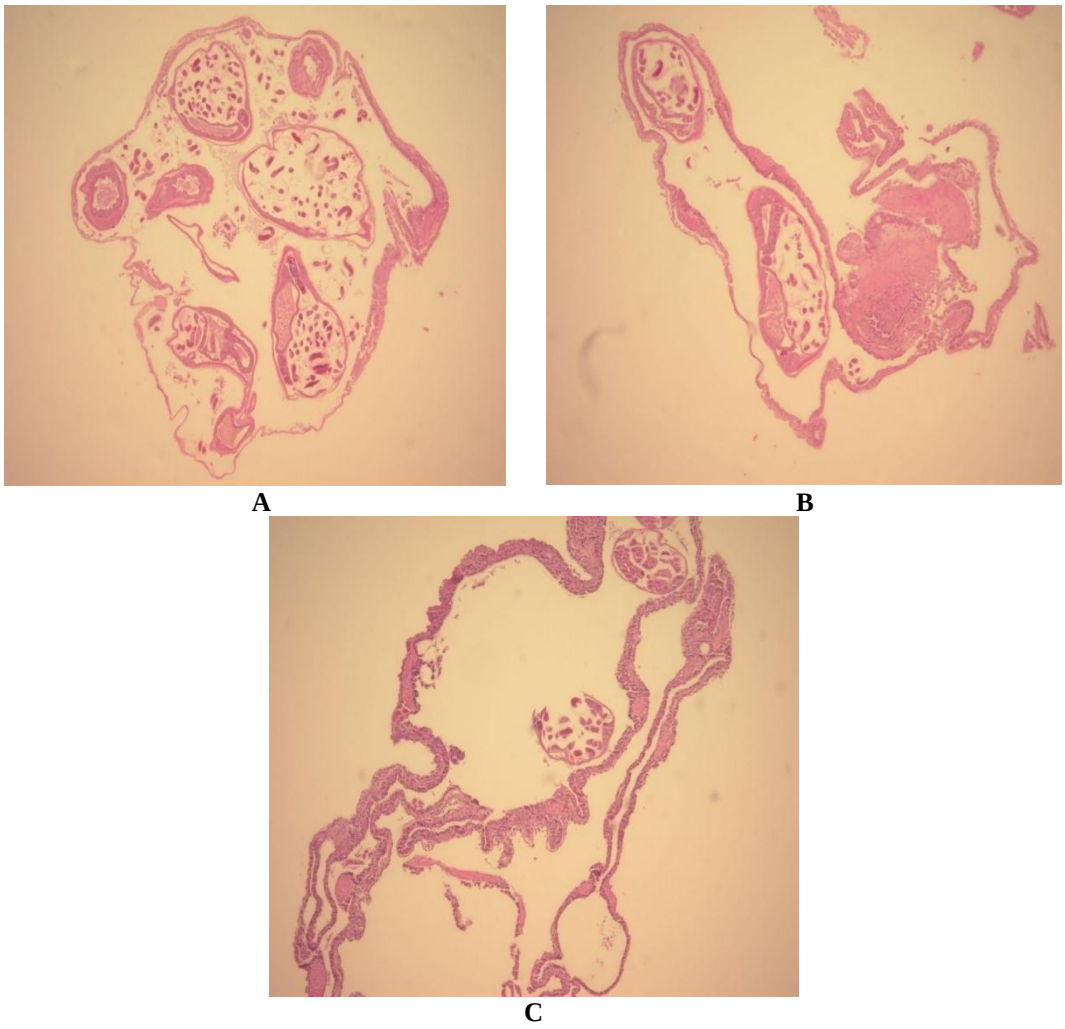
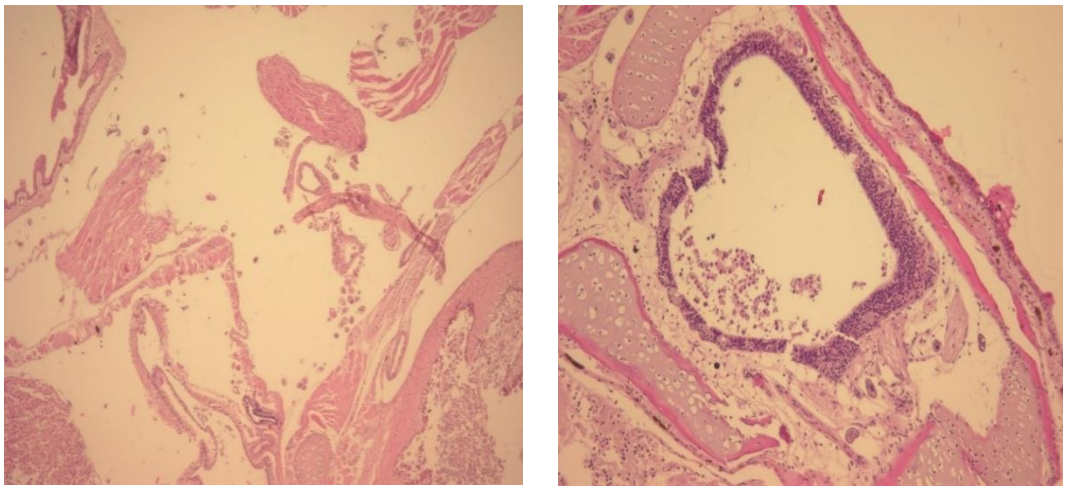
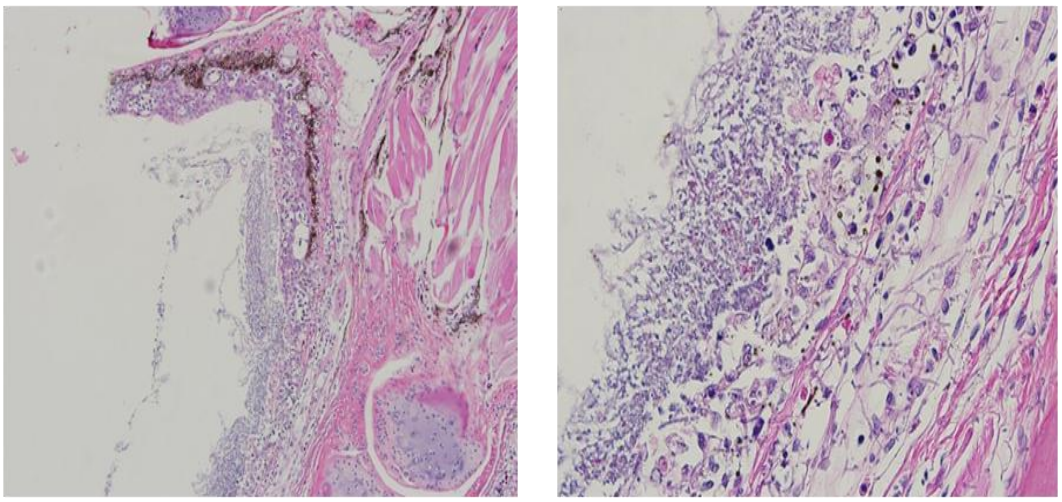


Figure 3: KST infected with lungworms. Parasites in the lung causing lung dilation (A), inflammatory reaction (B), and thickening of the epithelium (C).



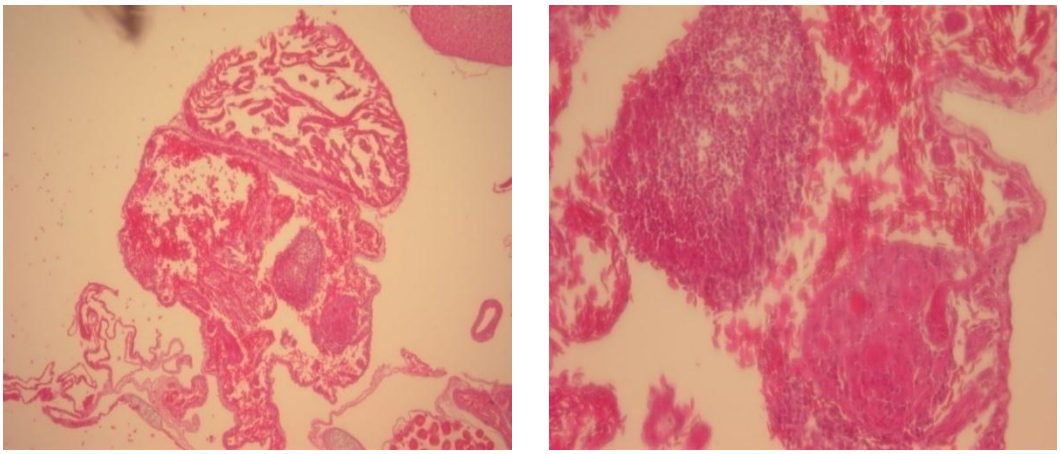
A **B**

Figure 4: Parasitic infections in KST. Ciliate parasites throughout the body.



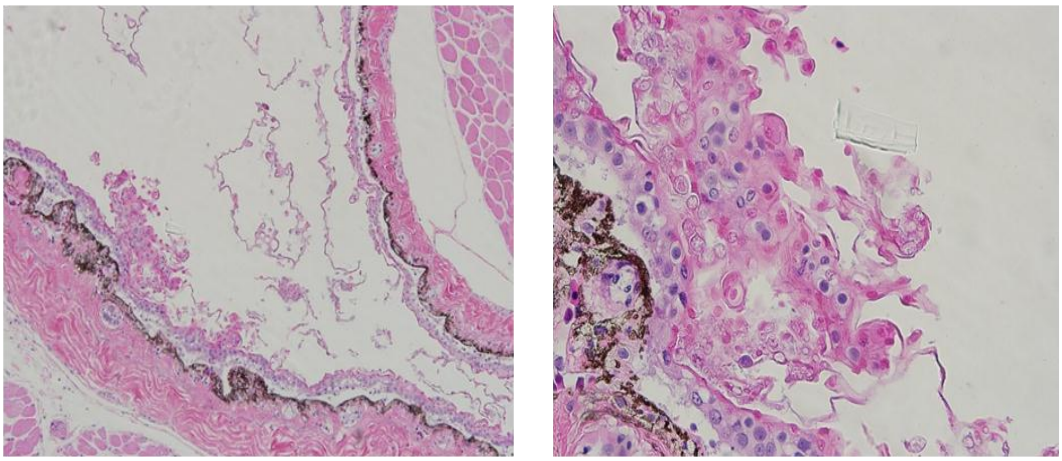
A **B**

Figure 5: KST skin. Inflammatory changes in response to bacterial and fungal infectionsin the feet. Note the fungal hyphae extending into the submucosa and infiltration of inflammatory cells.



A **B**

Figure 6: Heart of KST with endocarditis characterised with masses of reacting tissues and bacteria in one auricle.



A **B**

Figure 7: KST skin. Sloughing of the keratin layer due to chytridiomycosis (A).At higher magnification, the sloughed keratin is seen to have numerous circular empty structures that are typical empty chytrid sporangia (B).

The most commonly encountered non-infectious abnormality in KSTs was transformation of secretory cuboidal and columnar epithelium into keratinized squamous epithelium (squamous metaplasia). It was observed in 33.1% of the carcasses and it occurred along the epithelium lining, the oral and nasal mucosa, tongue, oesophagus, stomach, small and large intestines, and urinary bladder (Figure 8).

Figure 1 further shows that mixed disorders were observed in 9% of the carcasses. They included dual infections with strongyloides and bacteria (3.9%), lungworms (1.1%) or ciliates (1.1). Mixed strongyloides and squamous cell metaplasia disorders were observed in 2.2% of the carcasses, while triple infections with strongyloides, ciliates and bacteria were seen in 0.6% (Figure 1).

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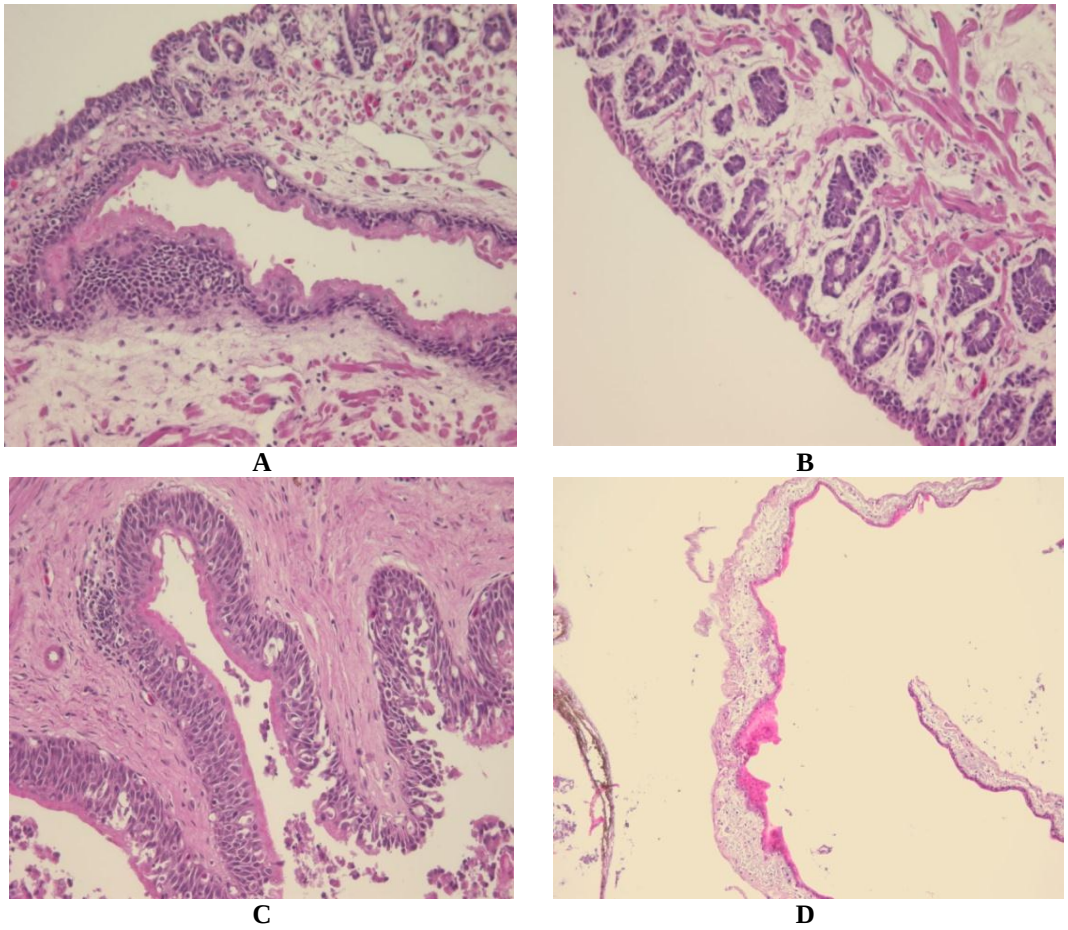

Figure 8: Sections of KST from various organs undergoing squamous metaplasia. A = ventral surface of the tongue showing non-glandular mucosa; B = dorsal surface of mucous secreting epithelial glands; C = small intestine; and D = colon.

Discussion

Keeping toads in biosecured captive facility prevents infections and drives the immune status to a basal non-stimulated state. This condition could create a new steady state naïve to antigenic challenges prevalent in the natural habitats. In fact, the captive bred KSTs may have parasite profile differing from their original parasite taxa. Re-introduction and subsequent interactions with other native fauna may cause host switching by parasites especially from native fauna to the re-introduced KSTs. This may have important consequences for the viability of the KSTs like survivorship, fecundity, and dispersal ability. It is estimated that the current KST to be re-introduced in the wild is a descendant of more than eight generations that have stayed in biosecured captivity for 16 years. This period could be enough to affect significantly the original immunity of the toads. No wonder helminths and chytrid fungus have always been fatal to KSTs in the captive facilities, while in some toads in the wild they have been tolerated.

Results presented in this work show that helminths, particularly nematodes, contribute largely to the infectious conditions affecting the KSTs in the captive facilities. Infections of frogs with nematodes have been observed by several other researchers. A study conducted by Men et al. (2016) on prevalence of internal helminth parasites in frogs, showed that 86.67% of the frogs were infected with parasites, of which 50% were nematodes. The majority (60.04%) were parasitic in the intestines, followed by urinary bladder (24.8%) and lungs (7.38%). Other studies have shown that toads can contain gastric-encysting nematodes and be heavily infected with rhabditoid lungworms (Lettoof et al. 2013, Kuzmin et al. 2016). The infective juveniles of the lungworm *Rhabdias* successfully develop between 5 and 35 °C (Langford and Janovy 2016). This range of temperature covers the KST's temperature of 18–20 °C making the KST a potential host for rhabdias lungworms.

The presence of parasites in frogs does not necessarily cause illness. Illness may develop when the number of parasites is overwhelmingly large, the animal is under nutritional deficiency or the immune system is compromised. Limited nutrition lessens host resistance to parasites and predisposes to illness. Studies have shown that a high resource diet enhances frog resistance to worm penetration and tolerance as worms travel to the gut and after establishment therein (Knutie et al. 2017c). In high resource diets, frogs infected with nematodes consume more food than non-parasitized frogs. If food is restricted, the masses of parasitized frogs decrease, while that of non-parasitized remain unchanged (Knutie et al. 2017c). This change may predispose to illness and death. In a recent study, a strong positive correlation between nematode prevalence and parasite numbers in frogs with Mn, Co, Ni, As, Se and Cd has been observed (De Donato et al. 2017). This correlation alludes further to the development of disease following nutritional deficiency.Experimental infection with Rhabdias (*Rhabdiaspseudosphaerocephala*) severely depresses the viability of metamorphs and survivorship of a tree frog, *Litoriasplendida* (Pizzatto and Shine 2011). The parasite also reduces the stamina of *L. splendid* and *L. caerulea* but does not reduce growth rates (Pizzatto and Shine 2011). Natural infections in captive bred Archey's frogs (*Leiopelmaarcheyi*) with nematodes like *Koerneria* sp. and *Rhabditis* sp. cause haemorrhagic purulent nasal discharge, weight loss and death (Shaw et al. 2011).

Infections of toads with chytrid fungus have been reported by others (Lettoof et al. 2013). We also experienced massive KSTs deaths in our captive facilities in 2012 due to chytrid outbreaks (Makange et al. 2014). These outbreaks have been controlled by re-enforcing our biosecurity procedures, work conduct and treatment with itraconazole (Makange et al. 2014). Chytridiomycosis remains to be a potential parasite as it can wipe up a population. In fact, it is strongly suggested that the extinction of the KSTs was partly contributed by chytrid fungus infections. The occurrence of chytrid fungus at Kihansi gorge and its surroundings (Makange et al. 2014) poses potential impending dangers to the re-introduced KSTs. To overcome this, a biological control study to make the KSTs tolerant to the fungus is underway.

Our finding on nutritional deficiency, particularly hypovitaminosis A and the resulting epithelial squamous metaplasia is in harmony with previous reports (Dugas et al. 2013, Brenes-Soto and Dierenfeld 2014, Pessier et al. 2014). Amphibians kept under captivity are constrained by nutrition, including vitamin A. Vitamin A is essential for cellular differentiation, morphogenesis, growth, vision, immune responses, and reproduction. Its deficiency compromises heavily the performance of frogs. A study reviewing postmortem findings in 167 frogs from 13 species kept in captivity identified epithelial squamous metaplasia due to vitamin A deficiency and polycystic nephropathy

presumably due to electrolyte imbalances as common problems (Pessier et al. 2014). Vitamin A deficiency occurs when animals are fed on insectivorous diet lacking the vitamin or carotenoids. It can be corrected by supplementing feeder insects such as crickets with provitamin A carotene (Brenes-Soto and Dierenfeld 2014). According to Dugas and colleagues, supplementing vitamin A has strong positive effects on the reproduction of captive frogs by improving the quality of fertilized eggs (Dugas et al. 2013). Supplementation through topical administration of vitamin A on an every other day or once a week achieves higher levels of the vitamin than standard nutrition to the frog (Sim et al. 2010). We dust insects with multivitamin preparations enriched with vitamin A twice a week in our captive facilities to control hypovitaminosis A.

Conclusions and recommendations

This study has highlighted some of the pathology encountered in the KSTs in our captive facilities. These diseases are mainly due to limited diversity of resources and genetics that most probably have reduced the immune responses to agents that would not be fatal in the wild. The control of these diseases is purely based on good management. In fact, at the moment all the disease conditions are controlled and the KSTs are in good health. They are routinely screened for internal parasites and dewormed every two months with mebendazole. Access to the biosecured facility is strictly controlled to authorized personnel. The stock is used as a source for the KSTs that are re-introduced to the Kihansi gorge periodically. Monitoring of the KST health is an ongoing exercise. It is recommended that more studies should be done to explore the lowered immunity in order to overcome fatalities when animals are re-introduced to their natural habitats where management such as treatment or deworming is difficult and not normally done.

References

Bataille A, Cashins SD, Grogan L, Skerratt LF, Hunter D, McFadden M, Scheele B, Brannelly LA, Macris A, Harlow PS, Bell S, Berger L and Waldman B 2015 Susceptibility of amphibians to chytridiomycosis is associated with MHC class II conformation. *Proc. Biol. Sci.* 282: pii:20143127. doi: 10.1098/rspb.2014.3127.

Brenes-Soto A and Dierenfeld ES 2014 Effect of dietary carotenoids on vitamin A status and skin pigmentation in false tomato frogs (*Dyscophusguineti*). *Zoo Biol.* 33: 544-552.

Channing A, Howell K, Loader S, Menegon M and Poynton J 2009 *Nectophrynoidesasperginis*. *IUCN Red List of Threatened Species*. Version 2011.2. International Union for Conservation of Nature.

De Donato C, Barca D, Milazzo C, Santoro R, Giglio G, Tripepi S and Sperone E 2017 Is trace element concentration correlated to parasite abundance? A case study in a population of the green frog *Pelophylax* synkl. *hispanicus* from the Neto River (Calabria, southern Italy). *Parasitol. Res.* 116: 1745-1753.

Demas GE 2004 The energetics of immunity: a neuroendocrine link between energy balance and immune function. *Horm. Behav*. 45: 173-180.

Dugas MB, Yeager J and Richards-Zawacki CL 2013 Carotenoid supplementation enhances reproductive success in captive strawberry poison frogs (*Oophagapumilio*). *Zool. Biol.* 32: 655-658.

Howick VM and Lazzaro BP 2014 Genotype and diet shape resistance and tolerance across distinct phases of bacterial infection. *BMC Evol. Biol.* 14:56.

Knutie SA, Shea LA, Kupselaitis M, Wilkinson CL, Kohl KD and Rohr JR 2017a Early-life diet affects host microbiota and later-life defenses against

parasites in frogs. *Integr. Comp. Biol.* 57: 732-742.

Knutie SA, Wilkinson CL, Kohl KD and Rohr JR 2017b Early-life disruption of amphibian microbiota decreases later-life resistance to parasites. *Nature Commun.* 8(1): 86. doi: 10.1038/s41467-017-00119-0).

Knutie SA, Wilkinson CL, Wu QC, Ortega CN and Rohr JR 2017c Host resistance and tolerance of parasitic gut worms depend on resource availability. *Oecologia* 183: 1031-1040.

Kuzmin Y, de Vasconcelos Melo FT, da Silva Filho HF and dos Santos JN 2016 Two new species of *Rhabdias* (Nematoda: Rhabdiasidae) from anuran amphibians in Pará, Brazil. *Folia Parasitologica* (Praha) 63: 015; doi: 10.14411/fp.2016.015

Langford GJ and Janovy J Jr 2016 Ecological factors responsible for the geographic distribution of *Rhabdias joaquinensis*: where do lungworms infect anurans in nature? *Parasitol. Res.* 115: 1305-1313.

Lettoof DC, Greenlees MJ, Stockwell M and Shine R 2013 Do invasive cane toads affect the parasite burdens of native Australian frogs? *Int. J. Parasitol.: Parasites Wildl.* 2: 155-164.

Lochmiller RL and Deerenberg C 2000 Trade-offs in evolutionary immunology: just what is the cost of immunity? *Oikos* 88: 87-98.

Makange M, Kulaya N, Biseko E, Kalenga P, Mutagwaba S and Misinzo G 2014 *Batrachochytrium dendrobatidis* detected in Kihansi spray toads at a captive breeding facility (Kihansi, Tanzania). *Dis. Aquat. Organ.* 111: 159-164.

Malago JJ and Nondoli H 2008 Sodium arsenite reduces severity of dextran sulfate sodium-induced ulcerative colitis in rats. *J. Zhejiang Univ. Sci. B* 9: 341-350.

Malago JJ and Sangu CL 2015Intraperitoneal administration of butyrate prevents the severity of acetic acid colitis in rats. *J Zhejiang Univ. Sci. B*16: 224-234.

Men Q, Han H, Zhao Q, Xia W, Dong H, Zhu S, Wang Z, Li C, Zhu X and Huang B 2016 The prevalence of helminth parasites in *Pelophylax nigromaculatus* (Anura: Ranidae) from Shanghai, China. *Acta Parasitol.* 61: 802-807.

Menegon M, Salvidio S and Loader SP 2004 Five new species of *Nectophrynoides* Noble 1926 (Amphibia Anura Bufanidae) from the Eastern Arc Mountains, Tanzania. *Trop. Zool.* 17: 97-121.

Pessier AP, Baitchman EJ, Crump P, Wilson B, Griffith E and Ross H 2014 Causes of mortality in anuran amphibians from an ex situ survival assurance colony in Panama. *Zoo. Biol.* 33: 516-526.

Pizzatto L and Shine R 2011 The effects of experimentally infecting Australian tree frogs with lungworms (*Rhabdias pseudosphaerocephala*) from invasive cane toads. *Int. J. Parasitol.* 41: 943-949.

Shaw S, Speare R, Lynn DH, Yeates G, Zhao Z, Berger L and Jakob-Hoff R 2011 Nematode and ciliate nasal infection in captive Archey's frogs (*Leiopelma archeyi*). *J. Zoo Wildl Med.* 42: 473-479.

Sim RR, Sullivan KE, Valdes EV, Fleming GJ and Terrell SP 2010 A comparison of oral and topical vitamin A supplementation in African foam-nesting frogs (*Chiromantis xerampelina*). *J. Zoo Wildl. Med.* 41: 456-460.

Sternberg ED, Lefèvre T, Li J, de Castillejo CLF, Li H, Hunter MD and de Roode JC 2012 Food plant derived disease tolerance and resistance in a natural butterfly–plant–parasite interactions. *Evolution: Int. J. Organic Evolut.* 66: 3367-3376.